Dual RAS blockade normalizes angiotensin-converting enzyme-2 expression and prevents hypertension and tubular apoptosis in Akita angiotensinogen-transgenic mice

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HYPERTENSION AFFECTS 25% OF the adult population in North America (1), and 40% of patients with diabetes develop hypertension (32). Hypertension and diabetes account for 65–70% of all end-stage renal disease (ESRD) cases in North America (1). ESRD is a major risk factor for cardiovascular complications, including myocardial infarction and stroke (8). While intensive insulin therapy and chronic treatment with renin-angiotensin system (RAS) blockers effectively retard the progression of diabetic nephropathy, they do not provide a cure (9, 14, 27, 28, 44). Such findings, however, indicate that hypertension, angiotensia, and RAS activation are major risk factors in the pathogenesis of ESRD. Human and murine renal proximal tubular cells (RPTCs) express all components of the RAS (19, 22, 34, 41). We have reported that transgenic (Tg) mice that specifically overexpress angiotensinogen (Agt), the sole precursor of angiotensins in RPTCs, develop hypertension, albuminuria, and tubular apoptosis (21, 30). Furthermore, Agt overexpression enhances tubular apoptosis in streptozotocin (STZ)-induced diabetic mice (20). Although these findings indicate that intrarenal RAS activation and hyperglycemia act in concert to enhance hypertension and RPTC apoptosis in diabetes, the molecular mechanism(s) underlying hypertension development and tubular apoptosis remain(s) incompletely understood.

Angiotensin-converting enzyme-2 (Ace2) shares 40–42% homology with angiotensin-converting enzyme (ACE) but possesses different biochemical activities (6, 35). Ace2 specifically cleaves ANG I and ANG II into ANG 1–9 and ANG 1–7, respectively. However, Ace2 has 400-fold higher catalytic efficiency on ANG II than on ANG I, resulting in ANG 1–7 formation (29, 38). Furthermore, identification of Mas as a receptor for ANG 1–7 established this heptapeptide as a biologically active member of the RAS cascade. ANG 1–7 opposes many ANG II-mediated actions, particularly vasoconstriction and vascular smooth muscle cell proliferation (31). Recently, administration of recombinant human Ace2 was reported to attenuate ANG II-dependent and pressure overload-induced hypertension and myocardial remodeling as well as renal injury in Ace2 knockout mice (42, 45, 46), further supporting an important counterregulatory role of Ace2 in ANG II-induced heart and renal disease.

The present study sought to determine whether a type 1 diabetic mouse model (Akita mice) in which rat Agt (rAgt) is overexpressed in the RPTCs would incur increased development of hypertension and nephropathy and whether RAS blockade could reverse these changes by normalizing renal Ace2 expression.

MATERIALS AND METHODS

Reagents. The following antibodies were used: polyclonal antibody against cleaved (active) caspase-3 (New England Biolabs, Pickering, ON), monoclonal anti-collagen type IV antibody (Chemicon International, Temecula, CA), polyclonal anti-ACE antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Ace2 antibody (R&D Systems, Minneapolis, MN), and monoclonal antibodies against β-actin (Sigma-Aldrich Canada, Oakville, ON). A rabbit polyclonal antibody

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against rAgt was generated in our lab (J. S. D. Chan) (39). This antibody is specific for intact rat and mouse Agt (55–62 kDa) and does not cross-react with pituitary hormone preparations or other rat or mouse plasma proteins (39). Albuminuria was detected by ELISA (Albuwell and Creatinine Companion, Exocell, Philadelphia, PA). pKAP2 plasmid containing the kidney-specific androgen-regulated protein (KAP) promoter responsive to testosterone stimulation was a gift from Dr. Curt Sigmund (University of Iowa, Iowa City, IA) and has been described elsewhere (5). Losartan (a nonpeptide ANG II-receptor subtype 1 blocker) and perindopril (an ACE inhibitor) were obtained from DuPont Merck (Wilmington, DE) and Servier Américue (Laval, QC), respectively. Oligonucleotides were synthesized by Invitrogen, (Burlington, ON). Restriction and modifying enzymes were procured from Invitrogen, Roche Biochemicals (Doral, QC) or GE Healthcare Life Sciences (Baie d’Urfé, QC).

**Generation of Akita AGT-Tg mice.** Tg mice (C57BL/6 background) that overexpress rAgt-HA [HA-tag, a sequence encoding amino acid residues 98–106 (YPYDVPDYA) of human influenza virus hemagglutinin] in RPTCs (line 388) driven by the KAP gene promoter were created in our laboratory (J. S. D. Chan) and have been described elsewhere (30). Homozygous Agt-Tg mice were then crossed with heterozygous Akita mice (C57BL/6-In2-kit2, Jackson Laboratories, Bar Harbor, ME; http://jaxmice.jax.org: N.B.: homozygous Akita mice are infertile). Breeding was continued until Akita Agt-Tg mice were obtained. These mice are homozygous for the rAgt transgene but heterozygous for insulin2 gene mutation. Akita Agt-Tg mice were identified by PCR of genomic DNA for the rAgt-HA transgene (30) and mutated insulin2 gene (43) (Table I). The DNA fragment (280 bp) of the mouse insulin2 gene was digested with the restriction enzyme Fnu4HI for 1 h at 37°C. If an allele was mutated, two DNA fragments of 280 and 140 bp were observed on 3% agarose gel electrophoresis.

**Physiological studies.** Male adult non-Akita littermates, Akita, Agt-Tg, and Akita Agt-Tg mice (8 mice/group) were studied. **group 1:** non-Akita littermates received vehicle intraperitoneally (ip); **group 2:** Akita mice were administered vehicle ip; **group 3:** Akita mice were given RAS blockers (losartan 30 mg·kg−1·day−1 plus perindopril 20 mg·kg−1·day−1) in drinking water from week 11 until week 16 (20, 30). **group 4:** A gt-Tg mice received vehicle ip; **group 5:** Akita A gt-Tg mice were administered vehicle ip; and **group 6:** Akita A gt-Tg mice were given RAS blockers as in **group 3.** All animals had ad libitum access to standard mouse chow and water. Animal care and procedures were approved by the Centre de Recherche, Centre Hospitalier de l’Université de Montréal (CRCHUM) Animal Care Committee.

Systolic blood pressure (SBP) was monitored in the morning with a BP-2000 tail cuff pressure monitor (Visitech Systems, Apex, NC) at least two to three times per week per animal for 8 wk (20, 21, 30). The mice were habituated to the procedure for at least 15–20 min/day for 5 days before the first SBP measurements. SBP values are expressed as means ± SE. All animals were housed individually in metabolic cages for 24 h before euthanasia at age 16 wk. Body weight was recorded. Urine was collected and assayed for albumin and creatinine by ELISAs (Albuwell and Creatinine Companion, Exocell) (20, 21, 30). Immediately after the animals were euthanized, the kidneys were excised (diced) and further increased in Akita A gt-Tg mice (Fig. 1B). A gt-Tg mice were obtained with extraction kits (Bachem Americas, Torrance, CA) according to the recommended number III protocol. The kidneys were then resuspended in 120 μl of EIA buffer solution (supplied by Bachem Americas), and aliquots (50 μl each) were taken for ANG 1–7 and ANG II measurement by respective specific ELISAs (Bachem Americas) (11).

**RESULTS**

**RPTC-specific expression of the Agt transgene in Akita and Tg mouse kidneys.** The mutated insulin2 gene was only detected in RPTs of Akita and Akita A gt-Tg mice but not in wild-type (WT) non-Akita or A gt-Tg mice (Fig. 1A). Similarly, the rAgt-HA transgene was expressed only in RPTs of A gt-Tg and Akita A gt-Tg mice but not in RPTs of WT and Akita mice (Fig. 1B). A gt expression in RPTCs of Akita mice (Fig. 1Cb) and A gt-Tg mice (Fig. 1Cc) was significantly higher than in WT mice (Fig. 1C) and further increased in Akita A gt-Tg mice (Fig. 1Cd) compared with Akita and A gt-Tg mice. These results confirm that the KAP gene promoter directs Agt transgene expression in RPTCs of A gt-Tg and Akita A gt-Tg mice.

**Physiological parameters in Akita and Tg mouse kidneys.** Average SBP began to rise in Akita and Akita A gt-Tg mice from week 9 compared with non-Akita WT mice, but no significant differences in SBP between Akita mice and Akita A gt-Tg mice were detected until week 12 (Fig. 2A). Similarly, no significant differences in SBP were observed between A gt-Tg mice and non-Akita WT mice until week 12 (Fig. 2A). Treatment with RAS blockers completely prevented the in-
crease in SBP in both Akita and Akita Agt-Tg mice (Fig. 2, A and B).

Blood glucose levels in untreated or RAS blocker-treated Akita and Akita Agt-Tg mice were significantly higher than in non-Akita WT and Agt-Tg mice, respectively (Fig. 3A). Kidney weight/body weight ratios were elevated in Akita and Akita Agt-Tg mice but not in Agt-Tg mice compared with non-Akita WT controls, and treatment with RAS blockers did not normalize these ratios (Fig. 3B). Importantly, the urinary albumin/creatinine ratio (ACR) was significantly higher in Akita, Agt-Tg, and Akita Agt-Tg mice than in non-Akita WT controls and was partially attenuated by treatment with RAS blockers (Fig. 3C). These data demonstrate that RAS blockers are effective in reducing albuminuria without affecting hyperglycemia and kidney hypertrophy in Akita and Akita Agt-Tg mice.

**Histological studies.** Unlike in WT non-Akita mice (Fig. 4Aa), renal structural damage was evident in Akita (Fig. 4Ab) and Akita Agt-Tg mice (Fig. 4Ad). Histological findings included tubular luminal dilatation, vascular degeneration in RPTCs, tubular luminal dilatation, and accumulation of cell debris in the tubular lumen. The kidneys of Akita Agt-Tg mice (Fig. 4Ad) showed more severe morphological changes, including marked tubular luminal dilatation, cell debris accumulation inside tubules, and loss of RPTC brush borders. Some RPTCs were even flattened. Treatment of Akita and Akita Agt-Tg mice with RAS blockers (Fig. 4, Ac and Af) strikingly suppressed, but did not completely reverse, these abnormalities.

Morphological analysis revealed significantly augmented tubular luminal area (Fig. 4B) and glomerular volume (Fig. 4C) in Akita and Akita Agt-Tg mice compared with non-Akita WT and Agt-Tg mice. Treatment with RAS blockers did not reverse the increases in tubular luminal area and glomerular volume in Akita or Akita Agt-Tg mice. In contrast, RPTC volume (Fig. 4D) was significantly greater in Akita, Agt-Tg, and Akita Agt-Tg mice than in non-Akita WT controls, and exposure to RAS blockers effectively overturned these changes.

**Ace2 and ACE expression in Akita and Tg mouse kidneys.** Immunostaining for Ace2 was decreased in RPTCs of Akita mice (Fig. 5Ab), Agt-Tg mice (Fig. 5Ad), and Akita Agt-Tg mice compared with WT non-Akita controls (Fig. 5Ac). RAS blockade normalized Ace2 immunostaining in RPTCs of Akita (Fig. 5Ac) and Akita Agt-Tg mice (Fig. 5Af). In contrast, RPTCs of Akita, Agt-Tg, and Akita Agt-Tg mice exhibited increased immunostaining for ACE (Fig. 5B, b, d, and e) compared with WT controls (Fig. 5Ba). RAS blockers decreased ACE immunostaining in RPTCs of Akita and Akita Agt-Tg mice (Fig. 5B, c and f) compared with Akita (Fig. 5Bb) and Akita Agt-Tg (Fig. 5Be). RT-qPCR for Ace2 and ACE mRNA expression (Fig. 5, C and D, respectively) and immunoblotting for Ace2 and ACE protein (Fig. 6, A and B, respectively) in isolated RPTs confirmed these findings.

Interestingly, urinary levels of ANG 1–7 were decreased, whereas ANG II levels were increased in Akita, Agt-Tg, and Akita Agt-Tg mice compared with non-Akita mice (Fig. 6, C and D, respectively). Treatment of Akita and Akita Agt-Tg mice with RAS blockers normalized urinary ANG 1–7 and ANG II levels.

**Tubulointerstitial fibrosis in Akita and Akita Agt-Tg mouse kidneys.** Masson’s trichrome staining and immunostaining for collagen type IV, respectively, revealed enhanced expression of collagenous components (Fig. 7A, b, d, and e) and collagen type IV (Fig. 7B, b, d, and e) in Akita, Agt-Tg, and Akita Agt-Tg mouse kidneys compared with non-Akita WT controls (Fig. 7, Aa and Ba). Once again, treatment with RAS blockers normalized staining for collagenous components and immunostaining for collagen type IV in Akita and Akita Agt-Tg mice (c and f in Fig. 7, A and B). Quantitative analysis of Masson trichrome staining in glomerulotubular (Fig. 7C) and glomerular areas (Fig. 7E) as well as collagen IV-immunostained images in glomerulotubular (Fig. 7D) and glomerular areas (Fig. 7F) confirmed these findings. The data indicate that RAS blockade effectively prevents interstitial fibrosis in Akita and Akita Agt-Tg mice.

**Tubular apoptosis in Akita and Tg mouse kidneys.** Next, we investigated the impact of RAS blockade on tubular apoptosis in Akita and Akita Agt-Tg mice. TUNEL assay disclosed positively stained nuclei in RPTCs of Akita mice (Fig. 8Ab)
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but not in non-Akita WT mice (Fig. 8Aa). Treatment with RAS blockers significantly prevented RPTC apoptosis in Akita mice (Fig. 8Ac). In contrast, tubular apoptosis was evident in Agt-Tg mice (Fig. 8Ad) and Akita Agt-Tg mice (Fig. 8Ae). Treatment with RAS blockers markedly attenuated but never completely reversed RPTC apoptosis (Fig. 8Af).

Expression of active caspase-3 was also enhanced in RPTCs from Akita, Agt-Tg, and Akita Agt-Tg mice (Fig. 8B, b, d, and e) compared with non-Akita littermates (Fig. 8Ba). Treatment with RAS blockers prevented active caspase-3 expression in Akita and Agt-Tg mice (Fig. 8B, c and f). These observations were confirmed by semiquantitation of TUNEL-positive RPTCs (Fig. 8C) and caspase-3 activity assays in RPTs (Fig. 8D). Treatment with RAS blockers significantly reduced the number of TUNEL-positive RPTCs (Fig. 8C) and caspase-3 activity (Fig. 8D) in both Akita and Akita Agt-Tg mice. The data demonstrate that RPTC apoptosis in Akita and Akita Agt-Tg mice can be prevented by RAS blockade.

Profibrotic and proapoptotic gene expression in Akita and Tg mouse kidneys. RT-qPCR disclosed increased TGF-β1 mRNA (Fig. 9A) and collagen type IV mRNA (Fig. 9B) expression in RPTs of Akita, Agt-Tg, and Akita Agt-Tg mice compared with non-Akita WT mice. Treatment with RAS blockers normalized their mRNA expression in Akita and Akita Agt-Tg mice (Fig. 9, A and B).

Bax mRNA expression was also significantly increased in RPTs of Akita, Agt-Tg, and Akita Agt-Tg mice compared with non-Akita WT mice (Fig. 9C). Treatment with RAS blockers inhibited Bax mRNA expression in Akita and Akita Agt-Tg mice. In contrast, expression of the antiapoptotic gene Bcl-xL was significantly lower in RPTCs from Akita, Agt-Tg, and Akita Agt-Tg mice than in non-Akita WT mice (Fig. 9D). RAS blockers normalized Bcl-xL mRNA expression in Akita and Akita Agt-Tg mice (Fig. 9D). These data indicate that RAS activation-induced RPTC apoptosis is mediated via enhanced Bax expression and decreased Bcl-xL expression in Akita, Agt-Tg, and Akita Agt-Tg mice.

DISCUSSION

Our data document that RAS blockade with an ACE inhibitor and an AT1R blocker effectively normalizes RPTC Ace2 expression and urinary ANG 1–7 level and prevents hypertension, albuminuria, tubulointerstitial fibrosis, tubular
apoptosis, and proapoptotic gene expression in RPTCs of Akita and Akita Agt-Tg mice, demonstrating a crucial role for intrarenal RAS activation in hypertension and tubular apoptosis in diabetes.

The Akita mouse is an autosomal dominant model of spontaneous type 1 diabetes in which the insulin gene is mutated. These mice exhibit decreased numbers of β cells of the pancreatic islets and develop hyperglycemia at age 3–4 wk (43). By age 30 wk, male Akita mice manifest impaired renal function with elevated serum IgA, glomerulosclerosis, and diffuse granular mesangial deposits of IgA as well as increases in oxidative stress markers in RPTs (13, 36) closely resembling those in type 1 diabetic patients. In the present study, the kidney weight/body weight ratios in Akita mice were elevated

Fig. 2. Systolic blood pressure (SBP) in male mice. A: longitudinal changes in mean SBP in male non-Akita WT (●), Akita (▲), Akita treated with renin-angiotensin system (RAS) blockers (●), Agt-Tg (▼), Akita Agt-Tg (○), and Akita Agt-Tg mice treated with RAS blockers (□). Baseline SBP was measured over a 5-day period before initiation of treatment. B: cross-sectional analysis of SBP (measured 2–3 times/animal per wk in the morning without fasting; week 16) in non-Akita WT, Akita, Agt-Tg, and Akita Agt-Tg mice treated with vehicle or RAS blockers in drinking water. Values are means ± SE; n = 8 (*P < 0.05, **P < 0.01, ***P < 0.005).

Fig. 3. Changes in mean blood glucose (A), kidney weight/body weight ratio (B), and urinary albumin/creatinine ratios (C) at week 16 in non-Akita WT, Akita, Agt-Tg, and Akita Agt-Tg mice. The animals were treated with vehicle or RAS blockers from week 11 to week 16. Values are means ± SE; n = 8. N.S., not significant. *P < 0.05. **P < 0.01. ***P < 0.005.
compared with non-Akita mice, as is characteristic of early type 1 diabetes.

Increased tubular cell apoptosis in diabetic kidneys has been documented (2, 3, 17, 18, 20, 33, 37). To further investigate the role of RAS activation in inducing renal injury, we created Akita Agt-Tg mice overexpressing rAgt specifically in their RPTCs. Consistently, the Agt-HA transgene was detected by RT-PCR in RPTs of Agt-Tg and Akita Agt-Tg mice but not in non-Akita and Akita mice. Agt protein expression (assessed by immunostaining) was significantly higher in RPTCs of Agt-Tg and Akita Agt-Tg than in non-Akita and Akita mice, respectively. These findings confirm that Agt expression is enhanced in RPTCs of Akita mice and that KAP directs Agt transgene expression in RPTCs of Tg mice (11, 20, 21, 30).

Longitudinal experiments revealed significantly higher baseline SBP in both Akita and Agt-Tg mice than in non-Akita control littermates, consistent with previous reports (11, 15, 20, 21, 26, 30). We detected further increases in SBP in Akita Agt-Tg mice compared with Akita and Agt-Tg mice. Interestingly, treatment of Akita and Akita Agt-Tg mice with RAS blockers normalized SBP, indicating the effectiveness of RAS suppression in preventing the development of hypertension in these mice. We found that dual RAS blockade was more effective in preventing the development of hypertension than monotherapy with either losartan or perindopril (data not shown). Thus we chose to study the effect of dual RAS blockade in the present study instead of losartan or perindopril alone. At present, little is known about the mechanisms that evoke elevated SBP in Akita mice. One possibility is that enhanced intrarenal RAS gene expression and intrarenal RAS activation leads to increased SBP. Heightened RPT Agt expression in Akita mice and prevention of hypertension by RAS blockade lend support to this notion.

Since microalbuminuria is an important clinical marker for the early detection of hypertension- or diabetes-induced nephropathy, we monitored urinary albuminuria with the ACR. We detected microalbuminuria in Akita, Agt-Tg, and Akita Agt-Tg mice at age 16 wk, and dual blockade of the RAS significantly reduced it. Taken together, these observations imply a link among renal RAS activation, hypertension, and albuminuria. However, it remains to be investigated whether albuminuria is secondary to elevated SBP or whether hyper-

Fig. 4. Morphological analysis of mouse kidneys at week 16. A: Hematoxylin-eosin staining of mouse kidneys. a: Non-Akita WT control littermate. b: Akita mouse. c: Akita mouse treated with RAS blockers. d: Agt-Tg mouse. e: Akita Agt-Tg mouse. f: Akita Agt-Tg mice treated with RAS blockers. Magnification ×600. B: mean tubular luminal area. C: mean glomerular volume. D: mean renal proximal tubule cell (RPTC) volume. Values are means ± SE; n = 8 (∗P < 0.05, **P < 0.01, ***P < 0.005).
tension and albuminuria are unrelated events in our Tg animal models.

The complete mechanism as to how dual blockade of the RAS normalizes elevated BP in Akita and Akita Agt-Tg mice is not clear. The hypothesis that ANG II downregulation of Ace2 gene expression subsequently facilitates the development of hypertension has received considerable attention. Indeed, RAS blockade was reported to increase cortical Ace2 activity and urinary ANG 1–7 excretion in normotensive Lewis rats (7). Koka et al. (16) observed low ACE and high Ace2 levels in normal human kidneys, with reversal of ACE and Ace2 expression in human hypertensive kidneys. Furthermore, they also determined that ANG II upregulated ACE and downregulated Ace2 expression in HK2 cells in vitro (16). We have reported markedly elevated renal ACE activity in Agt-Tg mice (11). Our present study demonstrated significantly lower ANG

Fig. 5. Expression of angiotensin-converting enzymes Ace2 and ACE in mouse kidneys at week 16. Shown is Ace2 (A) and ACE (B) immunostaining of mouse kidneys. Descriptions of a–f are the same as in Fig. 4. Magnification ×600. Arrows indicate cells stained positive for Ace2 (A) and ACE (B). Also shown is RT-qPCR of Ace2 (C) and ACE (D) in mouse RPTs. Ace2, ACE, and β-actin mRNAs were run simultaneously in RT-qPCR assay. Ace2 and ACE mRNA levels were normalized by corresponding β-actin mRNA levels. mRNA levels in non-Akita control littermates were considered as 100%. Values are means ± SE; n = 8 (∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.005).
1–7 and higher urinary ANG II levels in Akita, Agt-Tg, and Akita Agt-Tg mice than in non-Akita mice, and these changes were normalized by RAS blockers. Our findings lend additional support to the concept that RAS activation upregulates ACE activity and downregulates Ace2 expression. The precise mechanisms of RAS blockade-reversed downregulation of Ace2 and upregulation of ACE expression have yet to be investigated, although our previous study points to the involvement of ROS generation (11).

Histological examinations confirmed the presence of characteristic features of renal injury in the kidneys of Akita, Agt-Tg, and Akita Agt-Tg mice. Akita and Akita Agt-Tg mice exhibited RPTC hypertrophy, enhanced collagenous materials, and collagen type IV expression in glomeruli and the tubulo-interstitial space. The observations that RAS blockers completely prevented collagen type IV expression in Akita and Akita Agt-tg mice suggest a critical role of tubular RAS in the development of tubulointerstitial fibrosis in Akita mice.

Fig. 6. Western blotting of Ace2 and ACE in mouse kidneys and measurement of urinary ANG 1–7 and ANG II. Western blotting was done of Ace2 (A) and ACE (B) in mouse RPTs. The membranes were rebotted for β-actin. Urinary ANG 1–7 (C) and ANG II (D) levels are shown in non-Akita WT, Akita, Agt-Tg, and Akita Agt-Tg mouse kidneys at week 16. Peptides were extracted and assayed by specific ELISAs. Values are means ± SE; n = 8 (*P < 0.05, **P < 0.01, ***P < 0.005).
Fig. 7. Masson’s trichrome staining and immunostaining for collagen IV in mouse kidneys at week 16. A: Masson’s trichrome staining. B: immunostaining for collagen IV. Descriptions of a–f are the same as in Fig. 4. Magnification ×600. Quantifications of extracellular matrix component accumulation (Masson’s trichrome staining) in glomerulotubular area (C) and glomerular area (E) and of immunoreactive collagen IV deposition in glomerulotubular area (D) and glomerular area (F) are shown. Values are means ± SE; n = 8 (*P < 0.05, **P < 0.01, ***P < 0.005).
Fig. 8. Apoptosis in mouse kidneys at week 16, analyzed by terminal transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) staining (A), cleaved caspase-3 expression [4,6-diamidino-2-phenylindole (DAPI) staining; B], and caspase-3 activity assay. Descriptions of a-f are the same as in Fig. 4. TUNEL (green) and DAPI (blue) staining (∗×200) are shown for apoptotic cells and cellular nuclei, respectively. Arrows indicate TUNEL-positive cells in proximal tubules (A) and immunostaining for active caspase-3 staining (B). C: bar graph showing quantitative analysis of TUNEL-positive RPTC at week 16. D: caspase-3 activity assay in isolated RPTs at week 16. Values are means ± SE; n = 8 (∗P < 0.05, **P < 0.01, ***P < 0.005).
The precise mechanism(s) by which RAS activation causes interstitial fibrosis in Akita and Akita Agt-Tg mice remains unclear. One possibility is that ANG II elevation stimulates TGF-β1 and subsequently enhances the expression of extracellular matrix proteins, collagen type IV, and profibrotic and proapoptotic proteins in RPTCs, resulting in tubular injury (interstitial fibrosis and cellular apoptosis) (4). Indeed, neutralization of TGF-β1 alleviated fibrosis and tubular apoptosis in diabetic animal models (23, 47). Our present data showed higher TGF-β1 and collagen IV mRNA expression in RPTs of Akita, Agt-Tg, and Akita Agt-Tg mice than in non-Akita mice, and these changes were normalized by RAS blockade in Akita and Akita Agt-Tg mice. Taken together, these findings support a role for intrarenal RAS in interstitial fibrosis.

Consistent with our previous reports on the presence of TUNEL-positive cells and active caspase-3 expression in Agt-Tg and STZ-induced diabetic Agt-Tg mouse kidneys (11, 20, 21), we detected increases in TUNEL-positive RPTCs, active caspase-3 and Bax expression, and decreases in Bcl-xL expression in the RPTCs of Akita, Agt-Tg, and Akita Agt-Tg mice, which were reversed by treatment with RAS blockers. An elevated Bax/Bcl-xL ratio in Akita and Akita Agt-Tg mice is consistent with the promotion of tubular apoptosis, a potential mechanism by which RAS activation could enhance tubular apoptosis in Akita and Akita Agt-Tg mice.

Our present results may have obvious clinical implications for understanding type 1 diabetes. Since tubular apoptosis is detectable in human type 1 diabetic kidneys (17, 24, 25, 33), and tubular atrophy appears to be a better indicator of disease progression than glomerular pathology (10), we suggest that RPTC apoptosis may be an initial mechanism of tubular atrophy in diabetes. The RAS activation-mediated decrease of Ace2 gene expression would further accelerate this process.

Fig. 9. Transforming growth factor (TGF)-β1, collagen IV, and Bax and Bcl-xL mRNA expression in mouse kidneys at week 16. Shown is RT-qPCR of TGF-β1 (A), collagen IV (B), Bax (C), and Bcl-xL (D) mRNAs. TGF-β1, collagen type IV, Bax, Bcl-xL, and β-actin mRNAs were run simultaneously in RT-qPCR assays. TGF-β1, collagen IV, Bax, and Bcl-xL mRNA levels were normalized by corresponding β-actin mRNA levels. mRNA levels in non-Akita control littermates were considered as 100%. Values are means ± SE; n = 8 (*P < 0.05, **P < 0.01, ***P < 0.005).
In summary, the present study indicates a critical role of tubular RAS activation in the development of hypertension, albuminuria, tubulointerstitial fibrosis, and RPTC apoptosis in Akita mice. Our study also indicates that RAS blockade is effective in preventing or reversing these pathophysiological manifestations in type 1 diabetes.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


